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Purification and Characterization of Hyaluronidase from Oral *Peptostreptococcus* Species

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Hyaluronidase was purified to apparent homogeneity from the spent medium of *Peptostreptococcus* sp. strain 84H14S. The enzyme was purified 310-fold by ethanol precipitation, gel chromatography, and cation-exchange chromatography with a recovery of 42% of the original activity in the culture medium. The molecular weight of the purified enzyme was estimated to be 160,000 by gel filtration with Sephacryl S-300. Like bacterial mucopolysaccharidases of other sources, the enzyme carried out an eliminative reaction with the substrate, producing 4,5-unsaturated disaccharides as the final end products. Its optimum temperature of activity is 46°C. The purified peptostreptococcal hyaluronidase was different from previously reported bacterial hyaluronidases in several respects. It degraded hyaluronic acid rapidly and also exhibited some activity against chondroitin sulfate A and chondroitin sulfate C. The K_ms for hyaluronic acid, chondroitin sulfate A, and chondroitin sulfate C were 0.14, 1.4, and 2.6 mg/ml, respectively. The specific activity of hyaluronidase was much higher than that of any previously purified mucopolysaccharidases. The V_{max} against hyaluronic acid reached 400 mmol of product per min per mg of protein at 22°C. The peptostreptococcal hyaluronidase was also unique in that its optimum pH of activity was around neutrality, whereas other bacterial hyaluronidases were most active at acidic pHs.

There have been numerous reports on the production of hyaluronidases by oral bacteria (10, 16, 22, 25). Recently, with the anaerobic culture technique, hyaluronidase-producing peptostreptococci were found to be common inhabitants of the oral cavity of humans and were shown to be present in significantly higher numbers in the gingival crevices of diseased periodontium than in those of healthy periodontium (30). These organisms produced large amounts of extracellular enzyme in vitro (28). It is apparent that peptostreptococci are major contributors of the hyaluronidases present in dental plaque.

The production of hyaluronidases by oral bacteria has been thought to play a role in the pathogenesis of periodontal disease (1, 24). Bacterial hyaluronidases in the area of the gingival crevice may destroy an important component of the ground substance of connective tissue, leading to periodontal destruction on the one hand and enhancing the spread of bacterial toxins on the other. Partial breakdown of proteoglycan due to the penetration of small amounts of hyaluronidase may also expose cryptic antigenic determinants, resulting in destructive autoimmune reactions.

Peptostreptoccal hyaluronidase has never been purified and characterized. The characterization of this enzyme will help clarify its pathogenic role in periodontal and possibly other destructive connective tissue diseases. Purified hyaluronidases with different substrate specificities will serve as a useful tool in the structural analysis of mucopolysaccharides. This report describes the purification and characterization of hyaluronidase from *Peptostreptococcus* strain 84H14S.

MATERIALS AND METHODS

Materials. Bovine serum albumin fraction V, hyaluronic acid (grade I), chondroitin sulfate type A, chondroitin sulfate type B, chondroitin sulfate type C, heparin (grade I), testicular hyaluronidase, and chondroitinase ABC were from Sigma Chemical Co., St. Louis, Mo. Reagents for polyacrylamide gel electrophoresis, molecular weight protein standards for gel electrophoresis, Silver Staining Kit, and Bio-Rad Protein Assay dye were from Bio-Rad Laboratories, Richmond, Calif. Sephadex G-50, Sephacryl S-300, CM-Sepharose, and molecular weight protein standards for gel filtration were from Pharmacia, Uppsala, Sweden. Brain heart infusion broth and yeast extract were products of Difco Laboratories, Detroit, Mich. All other reagents were obtained from Fisher Scientific Co., Montreal, Quebec, Canada.

Bacterial strain and growth conditions. Sterile brain heart infusion broth supplemented with 0.5 g of yeast extract per 100 ml was prereduced by leaving it in an anaerobic chamber (with an atmosphere of 90% N_2, 5% H_2, and 5% CO_2) overnight. Peptostreptococcus strain 84H14S, isolated from a periodontal pocket of a petient with periodontitis (28), was inoculated into the broth and incubated at 37°C in the anaerobic chamber. Growth was monitored by the measurement of the optical density of a culture at a wavelength of 675 nm with a Gilford spectrophotometer (model Stasar II).

Protein determination. Protein was determined with the Bio-Rad Protein Assay dye, with bovine serum albumin fraction V as a standard.

Enzyme assays. During enzyme purification and the determination of the optimum temperature for activity, enzyme activity was measured by the turbidity reduction assay (29). A 1-ml volume of substrate solution (hyaluronic acid at 70 μg/ml) was incubated with 1 ml of enzyme solution in the presence of 0.05 M sodium phosphate buffer with 0.05 M NaCl (pH 7.0). At 10-min intervals, 0.4-ml samples of the assay mixture were withdrawn and mixed with 0.5 ml of acidified protein solution (1% [wt/vol] bovine serum albumin fraction V in 0.5 M sodium acetate buffer, pH 3.1) at room temperature. After maximum turbidity was reached (55

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TABLE 1. Purification of hyaluronidase from *Peptostreptococcus* sp. strain 84H14S

Purification step	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Yield (%)	Fold purification
Supernatant	200,000	80	2,500	100	1.0
Ethanol precipitation	120,000	5.0	24,000	60	9.6
Sephacryl S-300	100,000	0.44	230,000	50	91
CM-Sepharose	84,000	0.11	760,000	42	310

min), A_{400} was measured with a Gilford spectrophotometer (model Stasar II). One unit of activity was defined as the activity that reduced the absorbance by 0.1 in 30 min at 37°C, pH 7.0.

For the determination of the optimum pH of activity, substrate specificity, and enzyme kinetics, the enzyme was assayed by monitoring the increase in A_{232} due to the production of 4,5-unsaturated glucuronosyl residues (9). The enzyme was incubated with substrate in 0.05 M sodium phosphate buffer at 22°C, and the increase in absorbance with time was monitored in a Perkin-Elmer spectrophotometer (model 555) with a 1-cm light path. The blank contained heat-inactivated enzyme. The amount of unsaturated products was calculated from the increase in absorbance, with the use of 5.5 as the millimolar absorption coefficient (31).

Enzyme purification. One liter of stationary-phase culture was centrifuged at 8,000 × *g* for 30 min, and the supernatant containing extracellular hyaluronidase was used for further purification. Concentration was achieved by ethanol precipitation. The supernatant was cooled in an ice-salt bath to −5°C. Ethanol (95%) was cooled to −15°C with dry ice and added at the rate of ca. 10 ml/min with constant stirring to a final concentration of 33% ethanol by volume. The mixture was stirred slowly for another 20 min in the ice-salt bath and then centrifuged at 10,000 × *g*, at −5°C, for 30 min. The precipitate which contained most of the enzyme activity was suspended in 10 ml of 0.05 M sodium phosphate buffer (pH 6.0) with 0.05 M NaCl. The suspension was dialyzed against the same buffer at 4°C. To get rid of any undissolved debris, the suspension was centrifuged at 10,000 × *g* for 30 min, and the clear solution was applied to a Sephacryl (S-300 Superfine) column (2.5 by 98 cm) equilibrated with the same buffer. Elution was carried out at 4°C; 10-ml fractions were collected. Active fractions were pooled (100 ml) and applied to a column (1.5 by 15 cm) of CM-Sepharose, a cationic exchanger equilibrated with the same buffer, at room temperature. The column was washed with stepwise ionic-strength gradients produced by applying 0.075, 0.125, or 0.2 M NaCl to 100-ml volumes of buffer. Fractions (5 ml) were collected. Hyaluronidase activity was eluted with buffer at 0.2 M NaCl. Active fractions were pooled, dialyzed against distilled water at 40°C, and lyophilized.

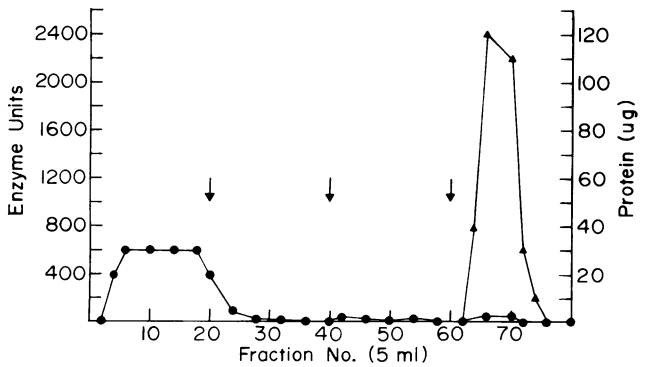

FIG. 1. Separation of peptostreptococcal hyaluronidase on a Sephacryl S-300 column (2.5 by 98 cm). Eluant was 0.05 M sodium phosphate buffer with 0.05 M NaCl (pH 6.0). Flow rate was 31 ml/h. Hyaluronidase activity was measured by the turbidity reduction assay, and protein was determined with the Bio-Rad Protein Assay dye. Symbols: ▲, enzyme; ●, protein.

Polyacrylamide gel electrophoresis. Polyacrylamide gels were run in a vertical gel electrophoresis cell (model 220; Bio-Rad). Electrophoresis was carried out under the nondenaturing condition devised by Furlong et al. (8), as described in the instruction manual for the vertical slab electrophoresis cell. A 10% final gel concentration was used. About 1 μg of the purified enzyme or 20 μg of the protein standards (composed of myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase) was loaded on each lane. Gels (0.75 mm thick) were electrophoresed at a constant current of 10 mA per gel until the tracking dye was near the bottom of the gel, and then the gels were stained with the Bio-Rad Silver Stain Kit according to Bio-Rad Laboratories bulletin 1089.

Molecular weight determination. Molecular weight was estimated on a Sephacryl S-300 (Superfine) column (2.5 by 98 cm) calibrated with catalase, aldolase, albumin, and ovalbumin from a Pharmacia calibration kit. The eluant was 0.05 M sodium phosphate buffer with 0.2 M NaCl (pH 6.0). Fractions of 2.1 ml were collected and assayed for protein and enzyme activity. Elution values were plotted on a selectivity curve against the logarithm of molecular weight by the method of Andrews (2).

Degradation of hyaluronic acid by peptostreptococcal hyaluronidase. Peptostreptococcal hyaluronidase (200 U) was incubated with 10 mg of hyaluronic acid in 10 ml of buffer (0.05 M sodium phosphate buffer, 0.05 M NaCl, pH 7.0) for 12 h at 37°C. Enzyme (200 U) was again added, and the mixture was incubated for an additional 12 h to ensure complete degradation. The mixture was then boiled for 10 min. The absorbance at different wavelengths (200 to 280 nm) of the boiled reaction mixture, appropriately diluted, was recorded with a Perkin-Elmer spectrophotometer (model 555) against an unreacted blank. A 5-ml volume of the boiled reaction mixture was fractionated on a Sephadex G-50 (Superfine) column (2.5 by 40 cm), with 0.2 M NaCl as eluant. Fractions of 2.1 ml were collected and assayed for uronic acid by the carbazole method (5). A 1-ml volume of sulfuric acid was added to 0.167 ml of each fraction, with cooling in an ice bath. The solution was thoroughly mixed and placed in a boiling water bath for 20 min. After the solution was cooled to room temperature, 0.33 ml of a 0.1% solution of carbazole in 95% ethanol was added. The solution was mixed and allowed to stand for 2 h before A_{530} was measured with a Gilford spectrophotometer (model Stasar II). The elution profile was compared with that produced by bovine testicular hyaluronidase and chondroitinase ABC from *Proteus vulgaris*.

Determination of optimum temperature for activity. An enzyme solution was appropriately diluted for assay at 18, 24, 28, 32, 36, 37, 42, 46, 50, and 56°C to find the optimum temperature for activity in the turbidity reduction assay (29).

Specificity against different substrates at different pHs. Equal concentrations (0.25 mg/ml) of hyaluronic acid, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, and heparin were incubated with the same amount (30 ng/ml) of enzyme at pHs of 5.6, 6.0, 6.4, 6.8, 7.0, 7.2, 7.6, 8.0, and 8.4 with 0.05 M sodium phosphate buffer. The rate of substrate degradation was measured by monitoring the increase of A_{232} at 22°C.

Enzyme kinetics. The K_m and V_{max} of the enzyme were determined for hyaluronic acid, chondroitin sulfate A, and chondroitin sulfate C from double-reciprocal (Lineweaver-Burk) plots of reaction velocity and substrate concentration. The rates of product formation were measured by the increase of A_{232} at 22°C.

RESULTS

Ethanol precipitation was an effective concentration and purification step for the hyaluronidase enzyme from the spent medium of *Peptostreptococcus* sp. strain 84H14S. A 10-fold purification was achieved with the recovery of 60% of the activity (Table 1). The hyaluronidase could be separated easily from most of the contaminating proteins, which had much smaller molecular weights, by gel chromatography (Fig. 1). Gel chromatography on Sephacryl S-300 effected a further 10-fold purification, with the recovery of over 80% of the enzyme activity loaded on the column. When the pooled fraction was applied to a cation-exchange CM-Sepharose column, the enzyme activity was retained by the column at low ionic strength while the majority of the proteins passed through the column (Fig. 2). Hyaluronidase activity was recovered by elution with 0.2 M NaCl buffer.

The final preparation contained 42% of the enzyme activity originally present in the supernatant. The hyaluronidase had been purified 310-fold. This preparation was shown to be homogeneous by gel electrophoresis (Fig. 3). The molecular weight of the purified enzyme was estimated to be 160,000 by gel chromatography (Fig. 4).

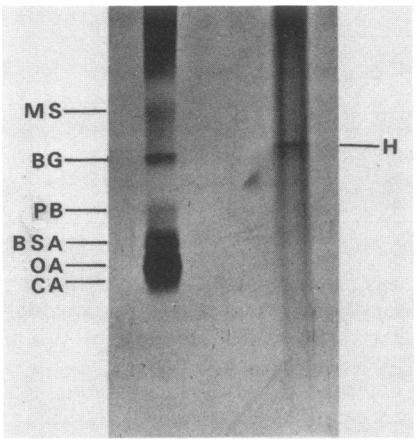

FIG. 3. Polyacrylamide gel electrophoresis of the purified enzyme in native state in a 10% gel system by the method of Furlong et al. (8). Gels were silver stained according to Bio-Rad Laboratories bulletin 1089. MS, Myosin (200 kilodaltons [K]); BG, β-galactosidase (116.25 K); PB, phosphorylase *b* (92.5 K); BSA, bovine serum albumin (66.2 K); OA, ovalbumin (45 K); CA, carbonic anhydrase (31 K); and H, hyaluronidase.

Peptostreptococcal hyaluronidase carried out an eliminative reaction with hyaluronic acid as the substrate, producing 4,5-unsaturated glucuronosyl residues, which absorb strongly around 230 nm. (For the enzyme and substrate concentrations used in all enzyme assays, the increase in absorbance with time was linear.) Disaccharides were the main end products of the reaction (Fig. 5). This pattern of degradation was similar to that of chondroitinase ABC of *Proteus vulgaris* but different from that of testicular hyaluronidase, which carried out a hydrolytic reaction that produced tetrasaccharides and hexasaccharides as the main products.

The optimum temperature of activity for the purified enzyme was ca. 46°C. The activity decreased rapidly at temperatures greater than 50°C.

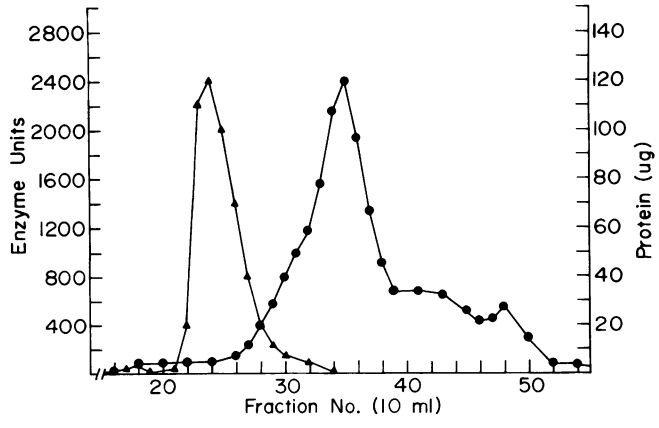

FIG. 2. Separation of peptostreptococcal hyaluronidase on a CM-Sepharose column (1.5 by 15 cm). The proteins were loaded in 0.05 M sodium phosphate buffer with 0.05 M NaCl (pH 6.0). Arrows (from left to right) indicate changes of eluting buffers with 0.075, 0.125, and 0.2 M NaCl. Hyaluronidase activity was measured by the turbidity reduction assay, and protein was determined with the Bio-Rad Protein Assay dye. Symbols: ▲, enzyme; ●, protein.

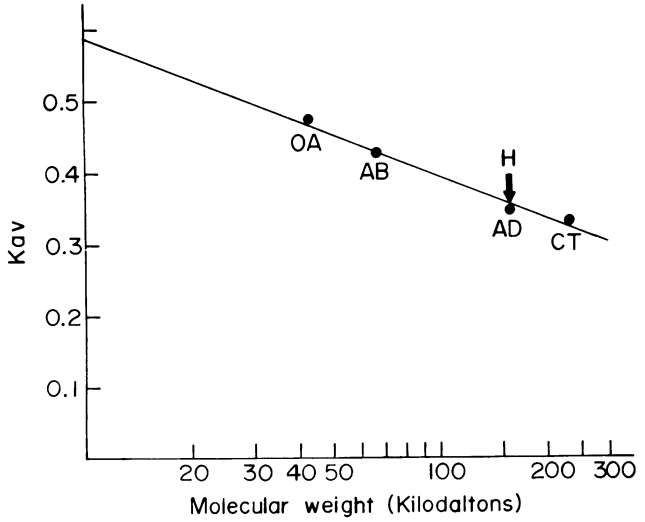

FIG. 4. Selectivity curve for the estimation of molecular weight by the method of Andrews (2) with a Sephacryl S-300 column (2.5 by 98 cm). CT, Catalase (232 K); AD, aldolase (158 K); AB, albumin (67 K); OA, ovalbumin (43 K); and H, hyaluronidase.

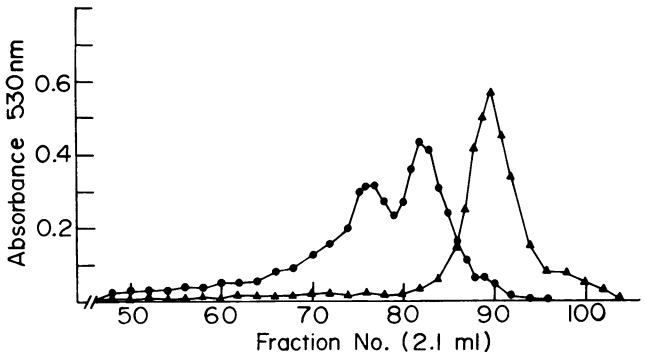

FIG. 5. Elution profile of the end products of degradation of 5 mg of hyaluronic acid by *Peptostreptococcus* hyaluronidase, *P. vulgaris*, and testicular hyaluronidase on a Sephadex G-50 column (2.5 by 40 cm). The A_{530} of each fraction, after reaction with carbazole, reflects the concentration of uronic acid. Symbols: ●, digested with testicular hyaluronidase; ▲, digested with *Peptostreptococcus* or *P. vulgaris* enzyme.

Peptostreptococcal hyaluronidase was highly active against hyaluronic acid over a wide pH range (Fig. 6). The enzyme was active from pH 6.4 to 7.6, with optimum activity at pH 7.2. With equal substrate concentrations, the activity against chondroitin sulfate A at pH 7.6 was about 1/10 that against hyaluronic acid at pH 7.2. The enzyme was active against chondroitin sulfate A over a wide pH range. This result was in contrast with that obtained when chondroitin sulfate C was the substrate; the enzyme was active against chondroitin sulfate C at pH 8.0. With equal substrate concentrations, the activity against chondroitin sulfate C at pH 8.0 was half that obtained with chondroitin sulfate A at pH 7.6. Over the pH range tested, the enzyme showed little or no activity against chondroitin sulfate B and heparin.

From double-reciprocal plots of reaction velocity and substrate concentration, the K_ms for hyaluronic acid, chondroitin sulfate A, and chondroitin sulfate C were 0.14, 1.4, and 2.6 mg/ml, respectively (Fig. 7 and 8). The estimated V_{max} for hyaluronic acid was 400 mmol/min per mg of protein at 22°C. The V_{max} for chondroitin sulfate A and chondroitin sulfate C was ca. 30 mmol/min per mg of protein at 22°C.

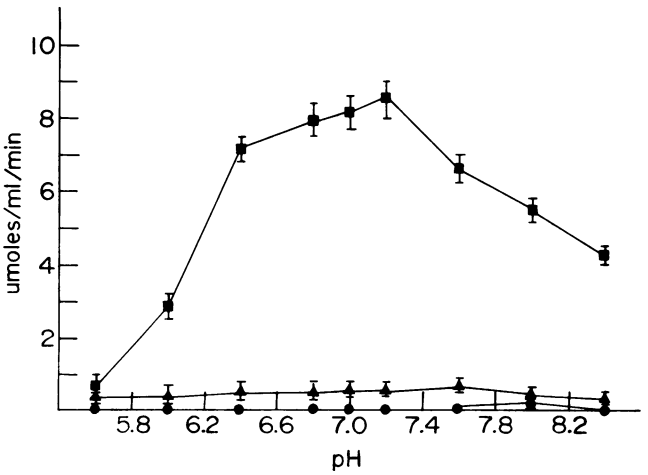

FIG. 6. Production of 4,5-unsaturated disaccharides at different pHs with different substrates by peptostreptococcal hyaluronidase. The reaction was carried out at 22°C. The reaction mixtures contained 30 ng of enzyme and 0.25 mg of substrate per ml of 0.05 M sodium phosphate buffer. The production of products per minute was monitored at A_{232}. At least three trials were made at each pH. Symbols: ■, hyaluronic acid; ▲, chondroitin sulfate A; ●, chondroitin sulfate C. With chondroitin sulfate B and heparin as substrates, no products were detected at the pHs tested.

DISCUSSION

Mucopolysaccharidases have been purified from animal, fungal, and bacterial sources. Bacterial mucopolysaccharidases differ from mucopolysaccharidases of other sources in that they produce 4,5-unsaturated disaccharides as the final products of the reaction (13, 31). *P. vulgaris* and *Bacteroides thetaiotaomicron* produce a chondroitinase ABC that acts mainly on chondroitin sulfates A, B, and C while showing little activity against hyaluronic acid (15, 31). *Flavobacterium heparinum* produces several mucopolysaccharidases (7, 17, 18, 26, 27, 31). Those that are relevant here include a chondroitinase AC that degrades chondroitin sulfates A and C but shows some activity against hyaluronic acid (31), a chondroitinase C that acts on chondroitin sulfate C and hyaluronic acid with almost equal efficiency (18), and a chondroitinase B that acts only on chondroitin sulfate B (17). Bacterial mucopolysaccharidases that act on hyaluronic acid have been purified only to a limited extent. These enzymes have been reported to act on hyaluronic acid only (6). These include enzymes from streptococci, clostridia, pneumococci (14), and *Escherichia* (4) and *Staphylococcus* species (21). The peptostreptococcal hyaluronidase reported here represents an enzyme of novel substrate specificity. It acts on hyaluronic acid at a rapid rate, whereas it degrades chondroitin sulfate A and chondroitin sulfate C at about 10 and 5%, respectively, the rate with hyaluronic acid. This activity against chondroitin sulfate A and chondroitin sulfate C was not due to a contaminating protein for two reasons.

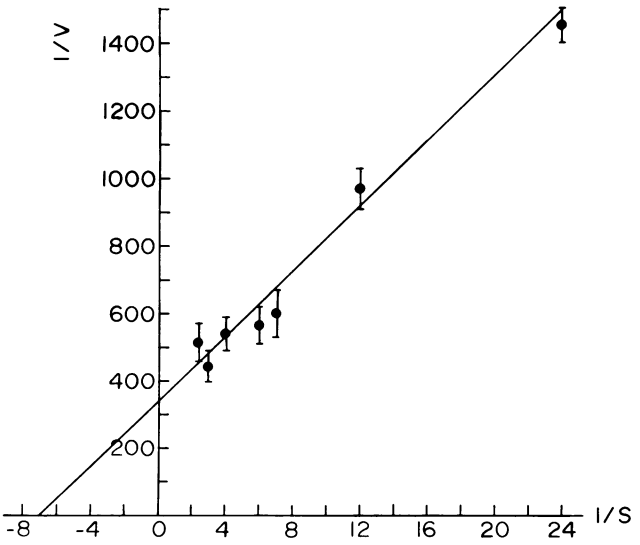

FIG. 7. Lineweaver-Burk plot of velocity versus substrate concentration for the purified hyaluronidase with hyaluronic acid as the substrate. The reaction was carried out at 22°C. The reaction mixture contained 6 ng of enzyme per ml in 0.05 M sodium phosphate buffer, pH 7.2. The production of products per minute was monitored at A_{232}. At least three determinations were made for each substrate concentration. The velocity, V, is expressed as millimoles of 4,5-unsaturated disaccharides formed per minute. The substrate concentration, S, is expressed in milligrams per milliliter.

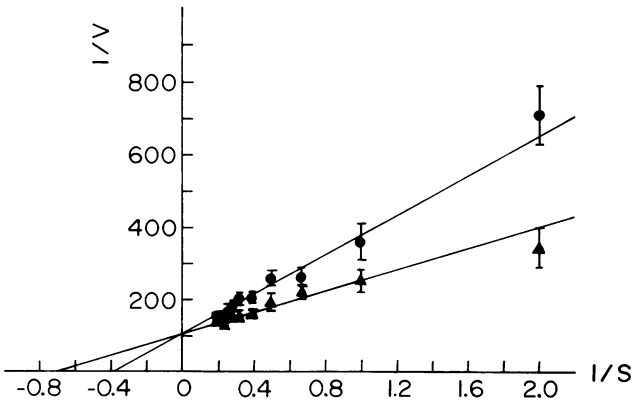

FIG. 8. Lineweaver-Burk plot of velocity versus substrate concentration for the purified hyaluronidase with chondroitin sulfate A (▲) and chondroitin sulfate C (●) as the substrates. The reaction was carried out at 22°C. The reaction mixture contained 300 ng of enzyme per ml in 0.05 M sodium phosphate buffer at pH 7.6 with chondroitin sulfate A as substrate and at pH 8.0 with chrondroitin sulfate C as substrate. The production of products per minute was monitored at A_{232}. At least three determinations were made for each substrate concentration. The velocity, V, is expressed as millimoles of 4,5-unsaturated disaccharides formed per minute. The substrate concentration, S, is expressed in milligrams per milliliter.

First, the purified enzyme was apparently homogeneous. Second, unpurified enzyme in the supernatant fraction showed the same rate of degradation with different substrates as the purified enzyme.

Commercial preparations of chondroitin sulfate A usually contain about 25% chondroitin sulfate C (23), and chondroitin sulfate C preparations may contain some chondroitin sulfate A. This mixture is due to the difficulty in separating the two mucopolysaccharides and sometimes the presence of both 4- and 6-sulfated disaccharides in the same molecule. Despite this situation, the observed chondroitin sulfate C lyase activity probably was not due to the activity against contaminating chondroitin sulfate A in the chondroitin sulfate C preparation because the pH optimum for chondroitin sulfate C activity is 8.0, wherease that for chondroitin sulfate A activity is 7.6. Furthermore, the K_m for chondroitin sulfate C (2.6 mg/ml) was only twice that observed for chondroitin sulfate A (1.4 mg/ml). The similarity of the V_{max}s for the two substrates may be a reflection of the cross-contamination of the two substrates.

Since the activity at 22°C was 60% of that at 37°C (data not shown), the calculated specific activity with hyaluronic acid as the substrate was 600 mmol/mg of protein at 37°C. This activity is 3 to 4 orders of magnitude higher than that of any previously purified mucopolysaccharidases (11, 31).

Peptostreptococcal hyaluronidase is also unique in that its optimum pH for activity is around neutrality. Other purified hyaluronidases are more active at acidic pHs (3, 4, 11, 12, 19, 20).

ACKNOWLEDGMENT

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LITERATURE CITED

1. **Aisenberg, M. S., and A. D. Aisenberg.** 1951. Hyaluronidase in periodontal disease. Oral Surg. **4**:317–320.
2. **Andrews, P.** 1965. The gel filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. **96**:595–606.
3. **Benchetrit, L. C., E. D. Gray, R. D. Edstrom, and L. W. Wannamaker.** 1978. Purification and characterization of a hyaluronidase associated with a temperate bacteriophage of group A, type 49 streptococci. J. Bacteriol. **134**:221–228.
4. **Brunish, R., and S. M. Mozersky.** 1958. The characterization of hyaluronidase isolated from *Escherichia freundii*. J. Biol. Chem. **231**:291–301.
5. **Davidson, E. A.** 1966. Analysis of sugars found in mucopolysaccharides. Methods Enzymol. **8**:52–60.
6. **Davidson, E. A., and K. Meyer.** 1954. Chondroitin, a new mucopolysaccharide. J. Biol. Chem. **211**:605–611.
7. **Dietrich, C. P., M. E. Silva, and Y. M. Michelacci.** 1973. Sequential degradation of heparin in *Flavobacterium heparinum*. J. Biol. Chem. **248**:6408–6415.
8. **Furlong, C., C. Cirakoglu, R. C. Willis, and R. A. Santy.** 1973. A simple preparative polyacrylamide disc gel electrophoresis apparatus: purification of three branched-chain amino acid binding proteins from *Escherichia coli*. Anal. Biochem. **51**:296–311.
9. **Greiling, H.** 1963. Hyaluronic acid, p. 87–90. *In* H. E. Bergmeyer (ed.), Methods of enzymatic analysis. Academic Press, Inc., New York.
10. **Hershon, L. E.** 1974. Elaboration of hyaluronidase and chondroitin sulfatase by micro-organisms inhabiting the gingival sulcus: evaluation of a screening method for periodontal disease. J. Periodontol. **42**:34–36.
11. **Hill, J.** 1976. Purification and properties of streptococcal hyaluronate lyase. Infect. Immun. **14**:726–735.
12. **Linder, L., C. E. Nord, and P. O. Soder.** 1971. Separation of hyaluronidase from a strain of anaerobic corynebacteria. Scand. J. Dent. Res. **79**:528–532.
13. **Linker, A.** 1966. Bacterial mucopolysaccharidases (mucopolysaccharide lyases). Methods Enzymol. **8**:650–654.
14. **Linker, A., K. Meyer, and P. Hoffman.** 1956. The production of unsaturated uronides by bacterial hyaluronidases. J. Biol. Chem. **219**:13–25.
15. **Linn, S., T. Chan, L. Lipeski, and A. A. Salyers.** 1983. Isolation and characterization of two chondroitin lyases from *Bacteroides thetaiotaomicron*. J. Bacteriol. **156**:859–866.
16. **Mahler, I. R., and V. G. Lisanti.** 1952. Hyaluronidase-producing microorganisms from human saliva. Oral Surg. **5**:1235–1240.
17. **Michelacci, Y. M., and C. P. Dietrich.** 1974. Isolation and partial characterization of an induced chondroitinase B from *Flavobacterium heparinum*. Biochem. Biophys. Res. Commun. **56**:972–980.
18. **Michelacci, Y. M., and C. P. Dietrich.** 1975. Chondroitinase C from *Flavobacterium heparinum*. J. Biol. Chem. **250**:1154–1158.
19. **Nord, C. E.** 1971. Purification and properties of hyaluronidase from *Streptococcus mitis*. Odontol. Revy **22**:125–136.
20. **Rautela, G. S., and C. Abramson.** 1973. Crystallization and partial characterization of *Staphylococcus aureus* hyaluronate lyase. Arch. Biochem. Biophys. **158**:687–694.
21. **Rogers, H. J.** 1954. The rate of formation of hyaluronidase, coagulase and total extracellular protein by strains of *Staphylococcus aureus*. J. Gen. Microbiol. **10**:209–220.
22. **Rosen, B., and N. B. Williams.** 1964. Hyaluronidase production of oral enterococci. Arch. Oral Biol. **9**:291–297.
23. **Salyers, A. A., and M. O'Brien.** 1980. Cellular location of enzymes involved in chondroitin sulfate breakdown by *Bacteroides thetaiotaomicron*. J. Bacteriol. **143**:772–780.
24. **Schultz-Haudt, S. D.** 1977. Biochemical aspects of periodontal disease. Int. Dent. J. **14**:398–406.
25. **Schultz-Haudt, S. D., and H. W. Scherp.** 1955. Production of hyaluronidase and beta-glucuronidase by viridans streptococci isolated from gingival crevices. J. Dent. Res. **34**:924–929.
26. **Silva, M. E., and C. P. Dietrich.** 1974. Isolation and partial characterization of three induced enzymes from *Flavobacterium heparinum* involved in the degradation of heparin and heparitin sulfates. Biochem. Biophys. Res. Commun. **56**: 965–972.
27. **Silva, M. E., and C. P. Dietrich.** 1975. Structure of heparin:

characterization of the products formed from heparin by the action of a heparinase and a heparitinase from *Flavobacterium heparinum*. J. Biol. Chem. **250**:6841–6846.

28. **Tam, Y.-C., and E. C. S. Chan.** 1983. Phase variation of hyaluronidase-producing peptostreptococci associated with periodontal disease. J. Dent. Res. **62**:1009–1012.
29. **Tam, Y.-C., and E. C. S. Chan.** 1983. Modifications enhancing reproducibility and sensitivity in the turbidimetric assay of hyaluronidase. J. Microbiol. Methods **1**:255–266.
30. **Tam, Y.-C., R. F. Harvey, and E. C. S. Chan.** 1982. Chondroitin sulfatase-producing and hyaluronidase-producing oral bacteria associated with periodontal disease. J. Can. Dent. Assoc. **48**:115–120.
31. **Yamagata, T., H. Saito, and S. Suzuki.** 1968. Purification and properties of bacterial chondroitinases and chondrosulfatases. J. Biol. Chem. **243**:1523–1535.